

The Absence of Activator Protein 1-dependent Gene Expression in THP-1 Macrophages Stimulated with Phorbol Esters Is Due to Lack of p38 Mitogen-activated Protein Kinase Activation*

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Activator protein 1 (AP-1) binds to the promoters of many genes involved in immune and inflammatory responses. We have previously shown that the p38 mitogen-activated protein (MAP) kinase regulates NF- κ B-dependent gene expression by modulating the phosphorylation and subsequent activation of TATA-binding protein (TBP). In this study, we asked whether the p38 MAP kinase regulated the transcriptional activity of AP-1. We found that phorbol 12-myristate 13-acetate (PMA) was unable to drive the AP-1-dependent reporter gene in THP-1 cells. PMA activated both the extracellular signal-regulated kinase and c-Jun NH₂-terminal kinase MAP kinases, but it did not activate the p38 MAP kinase. We found that cells expressing MAP kinase kinase 6(Glu), which is the upstream kinase that activates the p38 MAP kinase, had significantly increased AP-1-dependent gene expression alone and when stimulated with PMA. These cells also had increased phosphorylation of native c-Jun, suggesting that both c-Jun NH₂-terminal kinase and p38 MAP kinases phosphorylate c-Jun. More importantly, expression of a constitutive active MAP kinase kinase 6(Glu) resulted in the phosphorylation of a His-TBP fusion protein and increased direct interaction of TBP with c-Jun. These findings suggest that in macrophages, the p38 MAP kinase regulates AP-1-driven transcription by modulating the activation of TBP.

Activator protein 1 (AP-1)¹ is a transcription factor that binds to the promoters of genes, such as growth factors, chemokines, and cytokines, expressed in many immune and inflammatory diseases. AP-1 is a dimeric complex composed of members of the Fos and Jun proteins (1). These families of proteins are members of the basic leucine zipper group of proteins, and the basic leucine zipper motif mediates the for-

mation of homo- and heterodimers. The phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate is often used to induce AP-1 activity (2). AP-1 DNA binding does not always correlate with transcriptional activity. In fact, the induction of transcriptional activity requires the phosphorylation of c-Jun at N-terminal sites and c-Fos at C-terminal sites (3–8). In addition, it has been suggested that the interaction of all Jun family members and c-Fos and FosB with the basal transcription factors, transcription factor IIB (TFIIB) and TATA-binding protein (TBP), may be necessary for transcriptional activation (9–11).

The mitogen-activated protein (MAP) kinases are a family of second messenger kinases that are essential for transferring signals from the cell surface to the nucleus. Both the ERK and JNK MAP kinases have been linked to AP-1 activation (12–15). The ERK MAP kinase does not directly increase AP-1 transcriptional activity by phosphorylation of either c-Jun or c-Fos; rather, it phosphorylates Elk-1, which induces c-Fos synthesis (12, 16, 17). ERK MAP kinase also phosphorylates c-Jun at a C-terminal site, Ser-243, which actually inhibits its transcriptional activity (12). In contrast, the JNK MAP kinase phosphorylates c-Jun at the N-terminal sites Ser-63 and Ser-73, which increases its transcriptional activity (12, 17). The JNK MAP kinase, however, does not phosphorylate c-Fos. Unlike ERK or JNK, the p38 MAP kinase has been linked to AP-1 DNA binding but not phosphorylation of either c-Fos or c-Jun (18).

Based on our previous data showing that the p38 MAP kinase regulates NF- κ B-dependent gene expression by modulating the phosphorylation and subsequent activation of TBP (19, 20), we analyzed the role of the p38 MAP kinase in the regulation of AP-1-dependent gene expression in THP-1 cells. Utilizing a promoter construct driven only by AP-1, we found that phorbol 12-myristate 13-acetate (PMA) alone was unable to drive the AP-1-dependent reporter gene. PMA activated both the ERK and JNK MAP kinases, but it did not activate the p38 MAP kinase. We found that cells expressing a constitutive active MKK6(Glu), the upstream kinase of the p38 MAP kinase, had significantly increased AP-1-dependent gene expression in PMA-stimulated THP-1 cells, and SB 203580, a competitive inhibitor of p38, abrogated the effect of MKK6(Glu). AP-1 DNA binding was not altered by inhibition of the p38 MAP kinase, but the constitutive active MKK6(Glu) expression vector enhanced the phosphorylation of a His-TBP fusion protein. In addition, cells expressing MKK6(Glu) had increased direct interaction of TBP with c-Jun, whereas PMA alone had no significant effect on this interaction. We performed similar studies in RAW 264.7 macrophages, cells in which the p38 MAP kinase is activated by PMA. In these cells, AP-1-dependent gene expression was increased by PMA alone. These findings suggest that in macrophages, the p38 MAP kinase regulates AP-1-driven transcription by modulating the activation of TBP.

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¹ The abbreviations used are: AP-1, activator protein 1; TFIIB, transcription factor IIB; TFIID, transcription factor IID; TBP, TATA-binding protein; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH₂-terminal kinase; PMA, phorbol 12-myristate 13-acetate; MKK6, MAP kinase kinase 6; ATF, activating transcription factor; LPS, lipopolysaccharide; PAGE, polyacrylamide gel electrophoresis.

EXPERIMENTAL PROCEDURES

Cells—The THP-1 and RAW 264.7 cell lines were obtained from American Type Culture Collection (Manassas, VA). THP-1 cells were cultured in RPMI 1640 medium supplemented with gentamicin and 10% fetal calf serum (Life Technologies, Inc.). For *in vivo* phosphorylation studies, the cells were cultured in phosphate-free RPMI 1640 medium (Life Technologies, Inc.) with the same supplements, and for *in vivo* kinase assays, the cells were cultured in RPMI 1640 medium supplemented with gentamicin and 0.5% fetal calf serum. RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with gentamicin and 10% fetal calf serum.

Plasmids and Transfections—AP-1-dependent gene expression was evaluated using a luciferase reporter plasmid (pAP-1-luc) driven by six tandem copies of the AP-1 enhancer linked to a herpes simplex virus thymidine kinase promoter. The pcDNA-MKK6(Glu) and pCMV-Flag-p38 plasmids have been previously described (a generous gift from Dr. Roger Davis, University of Massachusetts, Worcester, MA) (21, 22). The pcDNA-His-TBP plasmid has been previously described (19). The pTET-luc and pTET-ATF plasmids were obtained from CLONTECH. Transfections were performed utilizing the Effectene transfection reagent (Qiagen, Valencia, CA), according to the manufacturer's instructions. Twenty-four h after transfection, the cells were stimulated with PMA (Sigma) at a dose of 100 ng/ml. Luciferase activity, which was normalized to total protein, was measured after 6 h, which was determined to be the time of maximal activity. When used, the p38 MAP kinase inhibitor SB 203580 (Calbiochem, La Jolla, CA) was used at 0.5 μ M and added 1 h prior to PMA.

Electrophoretic Mobility Shift Assays—THP-1 cells were stimulated with PMA for 3 h, and nuclear protein was extracted as previously described (23, 24). A consensus AP-1 (5'-CGCTTGATGAGTCAGCCG-GAA-3') oligonucleotide (Promega, Madison, WI) was labeled with [γ - 32 P]ATP, and binding reactions were performed as previously described (23, 24). Supershift assays were performed by incubating nuclear extracts with c-Fos or c-Jun rabbit polyclonal antibodies (Santa Cruz Biotechnology) 30 min prior to the binding reaction with the labeled oligonucleotide. Protein-DNA complexes were separated on a 5% polyacrylamide gel.

In Vitro and In Vivo Kinase Assays and Western Blot Analysis—Whole cell lysates were prepared as previously described (25, 26). The p38, JNK1, or ERK2 MAP kinases were immunoprecipitated from the lysates overnight at 4 °C with their respective rabbit polyclonal antibody (Santa Cruz Biotechnology) bound to Gammabind with Sepharose (Amersham Pharmacia Biotech). *In vitro* kinase activity was assayed as previously described using ATF-2, c-Jun, or TFIID (TBP) (Santa Cruz Biotechnology) as a substrate (19, 25, 26). Co-transfection of the pTRE-luc with the pTET-ATF plasmid was performed to determine p38 MAP kinase activity *in vivo*, according to the manufacturer's instructions. After 24 h, the cells were stimulated with PMA; luciferase activity, which was normalized to total protein, was measured after 6 h, which was determined to be the time of maximal activity. For Western blot analysis, SDS-polyacrylamide gels were transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech) as previously described (19). The p38, JNK1, and ERK2 MAP kinase rabbit polyclonal antibodies were used at a 1:1000 dilution, and the p-ERK monoclonal antibody (Santa Cruz Biotechnology) was used at a 1:500 dilution. The c-Jun and c-Fos rabbit polyclonal antibodies, the p-c-Jun monoclonal antibody, and the TFIID (TBP) rabbit polyclonal antibody (Santa Cruz Biotechnology) were used at dilutions of 1:150, 1:250, and 1:500, respectively. The AntiXpress monoclonal antibody (Invitrogen, Carlsbad, CA) recognizes the sequence Asp-Leu-Tyr-Asp-Asp-Asp-Lys from the leader peptide in the His-TBP fusion protein, and it was used at a 1:1000 dilution. Immunoreactive proteins were developed using a chemiluminescent (Amersham Pharmacia Biotech) or chemifluorescent (JBL Scientific, San Luis Obispo, CA) substrate.

In Vivo Phosphorylation—THP-1 cells were co-transfected with an empty vector or the pcDNA-MKK6(Glu) and the pcDNA-His-TBP plasmids. After 24 h, the cells were labeled with 32 P, as previously described (19). The His-TBP fusion protein was immunoprecipitated overnight at 4 °C from whole cell lysates with the AntiXpress monoclonal antibody bound to Gammabind with Sepharose. The samples were separated by SDS-PAGE, and phosphorylation was determined by autoradiography.

Statistical Analysis—All luciferase assays, which were normalized to total protein, are shown as means with the standard error. Statistical comparisons were performed using a paired, one-tailed *t* test, with a probability of $p < 0.05$ considered to be significant.

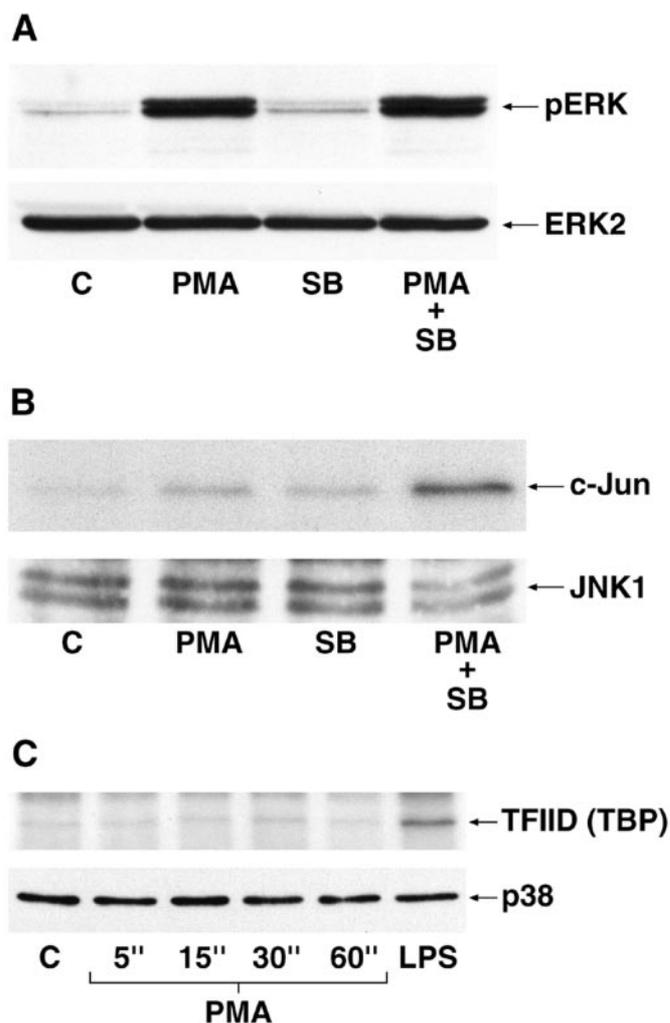


FIG. 1. Phorbol esters do not activate the p38 MAP kinase. A, cells were cultured in the presence or absence of SB 203580 (SB) and then exposed to PMA for 15 min. Whole cell lysates were separated by SDS-PAGE. Western blot analysis was performed using p-ERK monoclonal antibody at a 1:500 dilution and ERK2 rabbit polyclonal antibody at a 1:1000 dilution. C, cells were cultured as in A, and whole cell lysates were subjected to immunoprecipitation with JNK1 rabbit polyclonal antibody. *In vitro* kinase assays were performed using c-Jun as the substrate. Western blot analysis for JNK1 MAP kinase was performed to confirm equal loading of proteins. C, cells were exposed to PMA at the designated time points or to LPS for 15 min. Whole cell lysates were subjected to immunoprecipitation with the p38 MAP kinase rabbit polyclonal antibody. *In vitro* kinase assays were performed using TFIID (TBP) as the substrate. Western blot analysis for p38 MAP kinase was performed to confirm equal loading of the proteins.

RESULTS

PMA Activates Both the ERK and JNK MAP Kinases without Activating the p38 MAP Kinase—Because, in most cells, phorbol esters are strong activators of AP-1 activity, we first determined which MAP kinases were activated in THP-1 cells stimulated with PMA. ERK kinase activation was determined by performing a Western analysis for p-ERK. Cells were cultured in the presence or absence of SB 203580, the competitive inhibitor of the p38 MAP kinase, for 1 h and then stimulated with PMA for 15 min. We found that PMA strongly activated the ERK kinase and that SB 203580 had no effect on the status of p-ERK (Fig. 1A). Interestingly, PMA activated the ERK kinase as early as 5 min, and this activity stayed at the same level for as long as 60 min (data not shown).

JNK MAP kinase activity was determined by performing an *in vitro* kinase assay using c-Jun as the substrate. PMA activated the JNK1 MAP kinase in THP-1 cells, and SB 203580

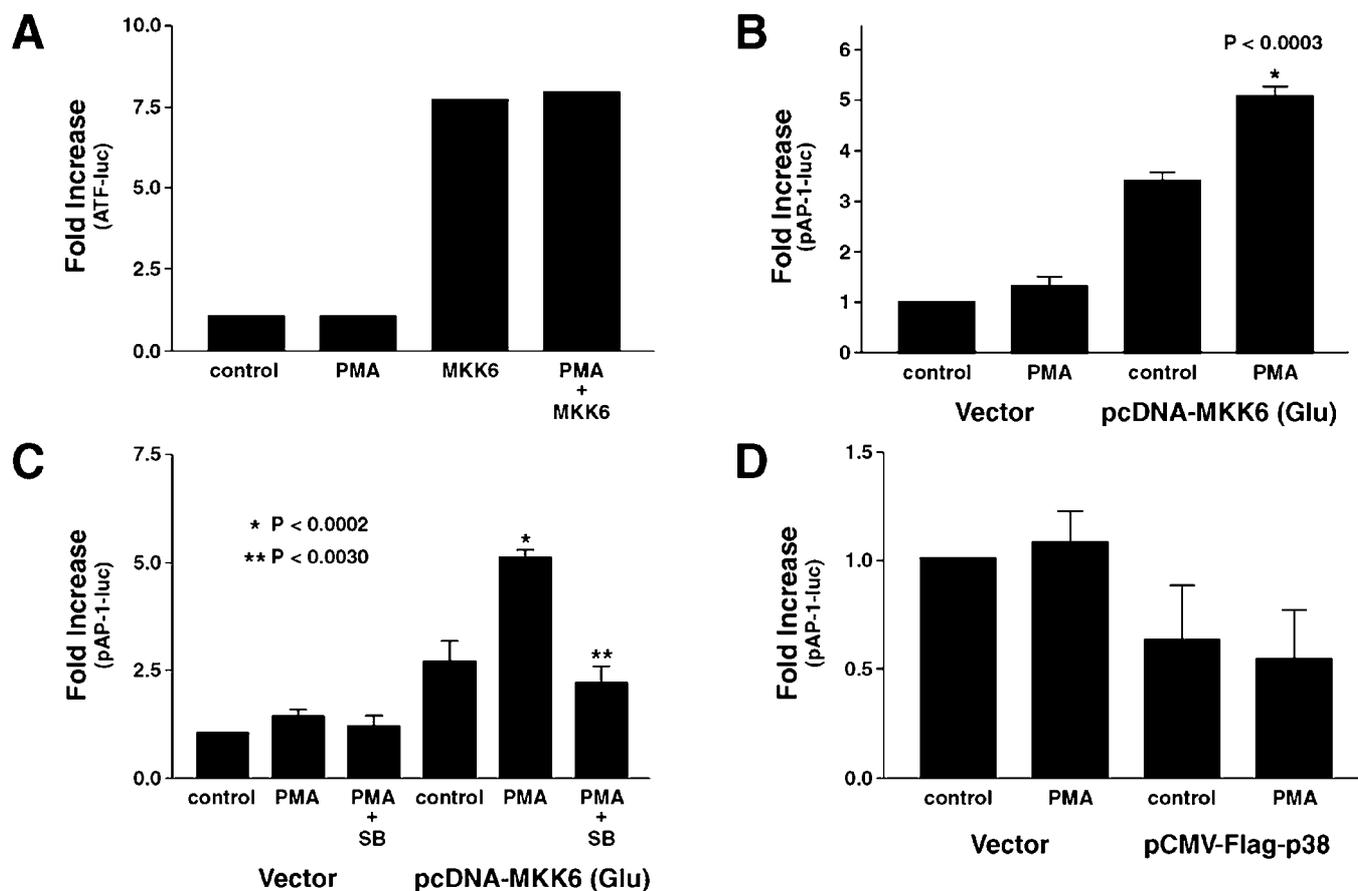


FIG. 2. An active p38 MAP kinase is necessary for AP-1-driven transcription. *A*, cells were transiently co-transfected with pTRE-luc, pTET-ATF and either an empty vector or the constitutive active MKK6(Glu) expression vector. *B*, cells were transiently co-transfected with the pAP-1-luc and either an empty vector or the constitutive active MKK6(Glu) expression vector. *C*, cells were transiently co-transfected as in *B* and then cultured in the presence or absence of SB 203580 (*SB*). *D*, cells were transiently co-transfected with pAP-1-luc and either an empty vector or the dominant negative p38 expression vector. In all experiments, the cells were exposed to PMA for 6 h the day after the transient transfection. All luciferase assays, which are normalized to total protein, are expressed as fold increase from control.

significantly augmented this activity as measured by the phosphorylation of c-Jun (Fig. 1*B*). In contrast, PMA did not activate the p38 MAP kinase, even over a prolonged period of time, as measured by *in vitro* kinase activity using TFIID (TBP) as a substrate. To assure ourselves that the p38 MAP kinase could be activated in these cells, we included LPS as a positive control (Fig. 1*C*). These data show that the ERK and JNK MAP kinases are activated in PMA-stimulated THP-1 cells, but the p38 MAP kinase is not activated. Furthermore, these data show that inhibition of the p38 MAP kinase with SB 203580 has no inhibitory effect on either ERK-mediated or JNK MAP kinase-mediated signaling. In fact, JNK MAP kinase-mediated signaling is enhanced by p38 MAP kinase inhibition.

A Constitutive Active MKK6 Is Necessary for AP-1-dependent Gene Transcription—To determine the role of the p38 MAP kinase in regulating AP-1-dependent transcription, we measured AP-1-dependent promoter activity using a luciferase reporter plasmid, pAP-1-luc. Because PMA did not activate the p38 MAP kinase, we utilized a constitutive active MKK6(Glu) plasmid to activate the p38 MAP kinase. We used an *in vivo* kinase assay to show that pcDNA-MKK6(Glu) was constitutively active in THP-1 cells. Cells were co-transfected with the luciferase reporter plasmid pTET-ATF and either an empty vector or the constitutive active MKK6(Glu) expression vector. In this assay, when the ATF is phosphorylated by the p38 MAP kinase via the constitutive active MKK6(Glu), the phosphorylated ATF can bind to the tetracycline-response element in the luciferase reporter promoter to induce luciferase expression. MKK6(Glu) alone and in combination with PMA increased

luciferase activity greater than 7.5 times the level of the control, whereas PMA alone had no effect on luciferase activity (Fig. 2*A*). An *in vitro* p38 MAP kinase assay using ATF-2 as a substrate revealed similar results (data not shown). These data confirm that pcDNA-MKK6(Glu) constitutively activates p38 both *in vivo* and *in vitro* and that PMA does not activate the p38 MAP kinase in THP-1 cells.

Utilizing the constitutive active MKK6(Glu) expression vector, we next evaluated its role in regulating AP-1-driven transcription. In these studies, we co-transfected pAP-1-luc with either an empty vector or the MKK6(Glu) expression vector. In cells expressing MKK6(Glu), there was a greater than 3-fold increase in luciferase activity in control cells and a greater than 5-fold increase in cells exposed to PMA. In contrast, PMA had no effect on luciferase activity in cells expressing the empty vector (Fig. 2*B*). A similar experiment was performed with cells cultured in the presence or absence of SB 203580 after the transient co-transfection. Cells expressing MKK6(Glu) had significantly increased luciferase activity compared with those expressing the empty vector, and SB 203580 abrogated the effect of MKK6(Glu) on AP-1-dependent luciferase activity (Fig. 2*C*).

To evaluate this in a different manner, cells were co-transfected with the pAP-1-luc plasmid and either an empty vector or the dominant negative pCMV-Flag-p38 plasmid. The results from these experiments corroborated the results found with SB 203580. PMA did not increase luciferase activity in cells expressing the empty vector, whereas luciferase activity in cells expressing the dominant negative p38 MAP kinase was below control levels (Fig. 2*D*). In aggregate, these results show that

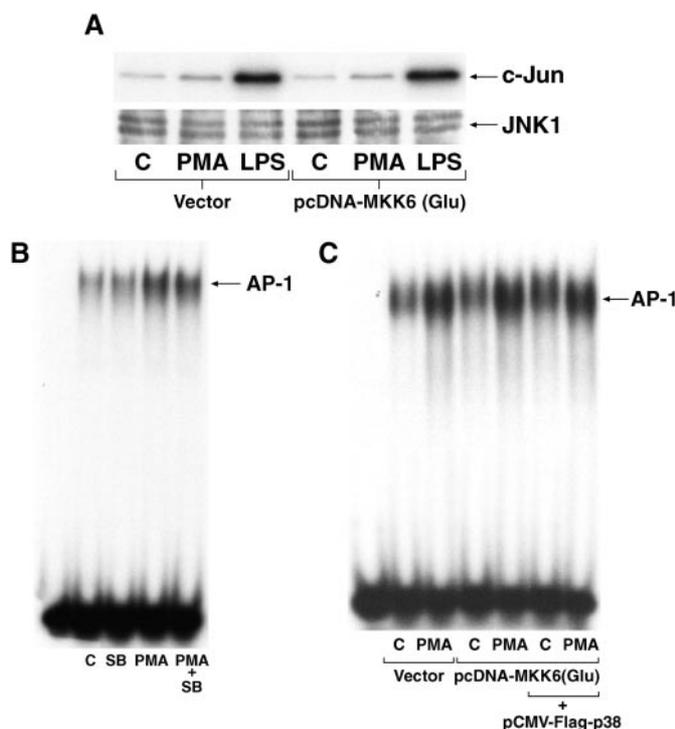


FIG. 3. The p38 MAP kinase does not regulate AP-1 DNA binding. *A*, THP-1 cells were transiently transfected with either an empty vector or pcDNA-MKK6(Glu). After 24 h, cells were exposed to PMA or LPS. Whole cell lysates were subjected to immunoprecipitation with the JNK1 MAP kinase rabbit polyclonal antibody. *In vitro* kinase assays were performed using c-Jun as the substrate. Western blot analysis for JNK1 was performed to confirm equal loading of the immunoprecipitated proteins. *B*, THP-1 cells were cultured in the presence or absence of SB 203580 (SB) and then exposed to PMA for 3 h. *C*, cells were transiently transfected with either an empty vector or the constitutive active MKK6(Glu) expression vector with or without the dominant negative p38. The total amount of plasmid DNA was equivalent in each sample by adding respective amounts of the empty vector. After 24 h, cells were stimulated with PMA for 3 h. In *B* and *C*, nuclear protein was isolated, and binding reactions were performed with a consensus AP-1 oligonucleotide labeled with [γ - 32 P]ATP.

AP-1-dependent gene expression, as measured by AP-1-dependent luciferase activity, requires an active p38 MAP kinase.

AP-1 DNA Binding Is Independent of p38 MAP Kinase Activation—In order to determine the role of p38 MAP kinase-mediated signaling in the regulation of AP-1-driven transcription, we first confirmed that the constitutive active MKK6(Glu) expression vector was not activating JNK MAP kinase-mediated signaling. Cells were transiently transfected with either an empty vector or pcDNA-MKK6(Glu). After 24 h, cells were treated with PMA or LPS, which was used as a positive control. Whole cell lysates were subjected to immunoprecipitation with JNK1 MAP kinase rabbit polyclonal antibody. *In vitro* kinase assays were performed using c-Jun as the substrate. We found that PMA slightly activated JNK1 compared with LPS in cells expressing the empty vector. There was no difference in this activity in cells expressing MKK6(Glu) (Fig. 3A). This validated the conjecture that the constitutive active MKK6(Glu) expression vector activated the p38 MAP kinase without altering JNK-mediated signaling.

We next determined the effect of the p38 MAP kinase on AP-1 DNA binding. Cells were cultured in the presence or absence of SB 203580 for 1 h and then stimulated with PMA for 3 h. An electrophoretic mobility shift assay showed that PMA increased DNA binding and SB 203580 had no significant effect on this binding (Fig. 3B). In a complementary experiment, cells were transiently transfected with either an empty vector or the constitutive active MKK6(Glu) with or without the dominant

negative p38 expression vector. After 24 h, the cells were stimulated with PMA for 3 h, and nuclear protein was extracted. An electrophoretic mobility shift assay showed that PMA increased DNA binding of AP-1, and there was no significant effect on this binding in cells expressing MKK6(Glu) with or without the dominant negative p38 (Fig. 3C). Utilizing a supershift assay, the AP-1 complex in PMA-stimulated THP-1 cells was composed of both c-Jun and c-Fos proteins (data not shown). These data suggest that the p38 MAP kinase does not regulate the binding of the AP-1 complex to DNA.

Due to the fact that the p38 MAP kinase did not regulate PMA-induced AP-1 DNA binding and the AP-1 complex contained both c-Jun and c-Fos, we next determined the effect of PMA on the nuclear accumulation of c-Jun and c-Fos proteins. Cells were cultured in the presence or absence of PMA for 3 h, and nuclear protein was extracted. Nuclear extracts were separated by SDS-PAGE, and a Western blot analysis was performed for either c-Jun or c-Fos. PMA did not increase the accumulation of c-Jun protein (Fig. 4A), but it significantly increased the amount of c-Fos protein (Fig. 4B). These data suggest that c-Jun is constitutively present in the nucleus of THP-1 cells and that PMA increases synthesis and translocation of c-Fos. The translocation of c-Fos to the nucleus allows it to combine with c-Jun to form an AP-1 complex that is more stable than those formed by Jun proteins alone (27).

Because the p38 MAP kinase does not regulate AP-1 DNA binding and PMA did not affect c-Jun translocation to the nucleus, we next determined whether p38-mediated signaling was involved in c-Jun phosphorylation. THP-1 cells were transfected with either an empty vector or the constitutive active MKK6(Glu) expression vector. After 24 h, cells were stimulated with PMA for 3 h, and nuclear protein was extracted. Nuclear extracts were separated by SDS-PAGE, and a Western blot analysis was performed for native p-c-Jun. In cells expressing the empty vector, PMA significantly increased the phosphorylation of c-Jun, whereas in cells expressing MKK6(Glu), there was a slight increase in the amount of phosphorylated c-Jun in both control cells and in PMA-stimulated cells (Fig. 4C). This finding shows that PMA, through activation of the JNK kinase, stimulates the phosphorylation of c-Jun and that MKK6(Glu), through activation of the p38 MAP kinase, also stimulates the phosphorylation of c-Jun. In aggregate, these data also show one mechanism by which control cells expressing MKK6(Glu) increase AP-1-dependent gene transcription. Furthermore, these data suggest that cells expressing MKK6(Glu) and stimulated with PMA have enhanced AP-1-dependent gene transcription due to the fact that PMA augments AP-1 DNA binding.

TBP Activation Is Required for AP-1-dependent Transcription—Based on our previous studies demonstrating the role of the p38 MAP kinase in regulating TBP phosphorylation and subsequent activation (19, 20), we next evaluated the role of TBP in AP-1-driven transcription. We first determined whether expression of the constitutive active MKK6(Glu) expression vector resulted in increased TBP phosphorylation. THP-1 cells were transiently co-transfected with pcDNA-His-TBP and either an empty vector or the MKK6(Glu) expression vector. After 24 h, the cells were labeled with 32 P_i in phosphate-free medium, washed, and subsequently exposed to PMA for 1 h. Whole cell lysates were subjected to immunoprecipitation with AntiXpress monoclonal antibody, and samples were separated by SDS-PAGE. We found that PMA had no significant effect on TBP phosphorylation *in vivo* in cells expressing the empty vector. In contrast, THP-1 cells expressing MKK6(Glu) had approximately a 3-fold increase in phosphorylation of TBP, as measured by densitometry (Fig. 5A). A Western blot analysis was performed for His-TBP

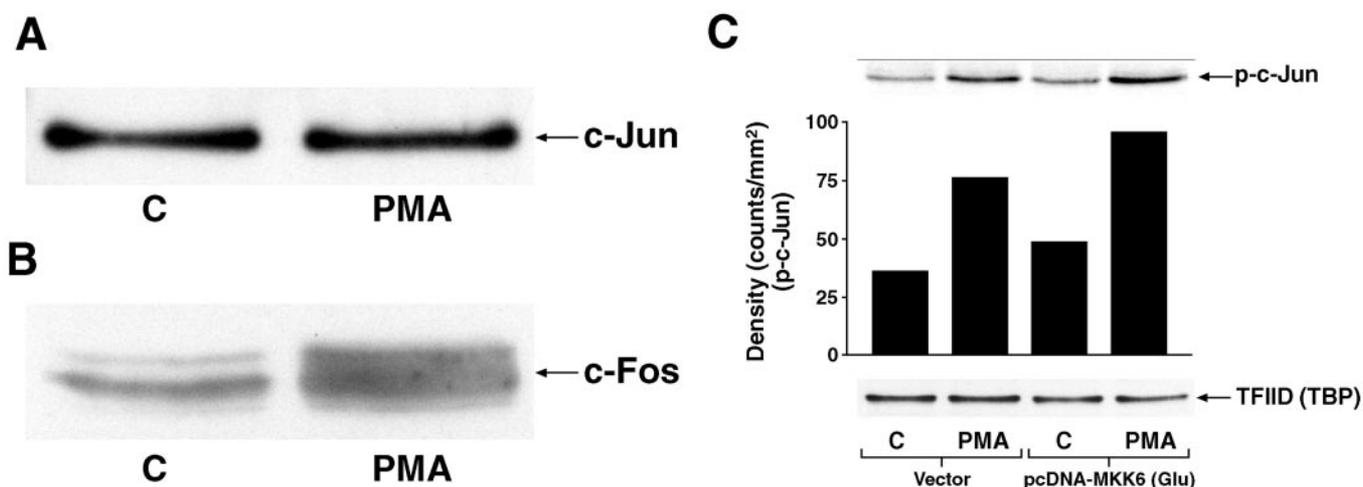


FIG. 4. Both JNK and p38 MAP kinases phosphorylate c-Jun. *A*, THP-1 cells were cultured in the presence (*PMA*) or absence (*C*, control) of PMA for 3 h. Nuclear protein was isolated, and samples were separated by SDS-PAGE. Western blot analysis was performed using the c-Jun rabbit polyclonal antibody at a 1:150 dilution. *B*, cells were cultured as in *A*. Western blot analysis was performed using the c-Fos rabbit polyclonal antibody at a 1:150 dilution. *C*, THP-1 cells were transiently transfected with either an empty vector or pcDNA-MKK6(Glu). After 24 h, the cells were stimulated with PMA for 3 h. Nuclear protein was isolated, samples were separated by SDS-PAGE, and Western blot analysis was performed with p-c-Jun monoclonal antibody at a 1:250 dilution. Densitometry of phosphorylated c-Jun is expressed in counts/mm². Western blot analysis for nuclear TFIID (TBP) was performed to confirm equal loading of the proteins.

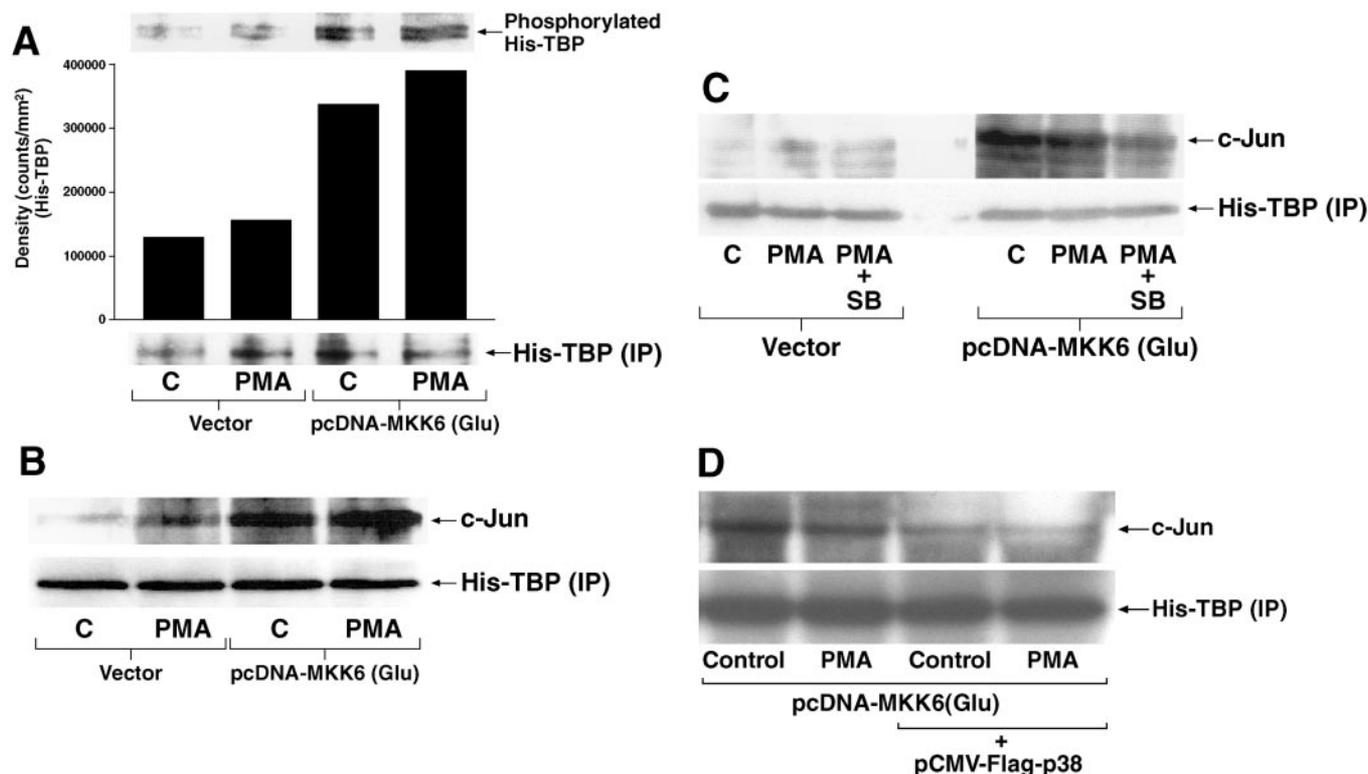


FIG. 5. The constitutive active MKK6 results in the phosphorylation of TBP and increases direct interaction of TBP and c-Jun. *A*, THP-1 cells were transiently co-transfected with pcDNA-His-TBP and either an empty vector or the constitutive active MKK6(Glu) expression vector. After 24 h, the cells were labeled with ³²P_i in phosphate-free medium for 3 h and then exposed to PMA for 1 h. Whole cell lysates were subjected to immunoprecipitation with AntiXpress monoclonal antibody, and samples were separated by SDS-PAGE. Densitometry of phosphorylated His-TBP is expressed in counts/mm². *B*, cells were transiently co-transfected as in *A*. After 24 h, the cells were exposed to PMA for 1 h. *C*, cells were transiently co-transfected as in *B* and then cultured in the presence or absence of SB 203580. *D*, cells were transiently co-transfected with pcDNA-His-TBP and the constitutive active MKK6(Glu) expression vector either alone or with the dominant negative pCMV-Flag-p38 plasmid. The total amount of plasmid DNA was equivalent in each sample by adding respective amounts of the empty vector. In *B-D*, whole cell lysates were subjected to immunoprecipitation with AntiXpress monoclonal antibody, samples were separated by SDS-PAGE, and Western blot analysis was performed with c-Jun rabbit polyclonal antibody at a 1:250 dilution. Western blot analysis for His-TBP was performed in *A-D* to confirm equal loading of the immunoprecipitated proteins.

to confirm equal loading of the immunoprecipitated proteins. These data show that cells expressing the constitutive active MKK6(Glu) have increased phosphorylation of TBP *in vivo*.

One study has shown that c-Jun directly interacts with the C terminus of TBP (11), so we determined whether the constitu-

tive active MKK6(Glu) increased this direct interaction in THP-1 cells. Cells were transiently co-transfected as in the *in vivo* phosphorylation experiments described in the preceding paragraph. After 24 h, cells were exposed to PMA, and whole cell lysates were subjected to immunoprecipitation with Anti-

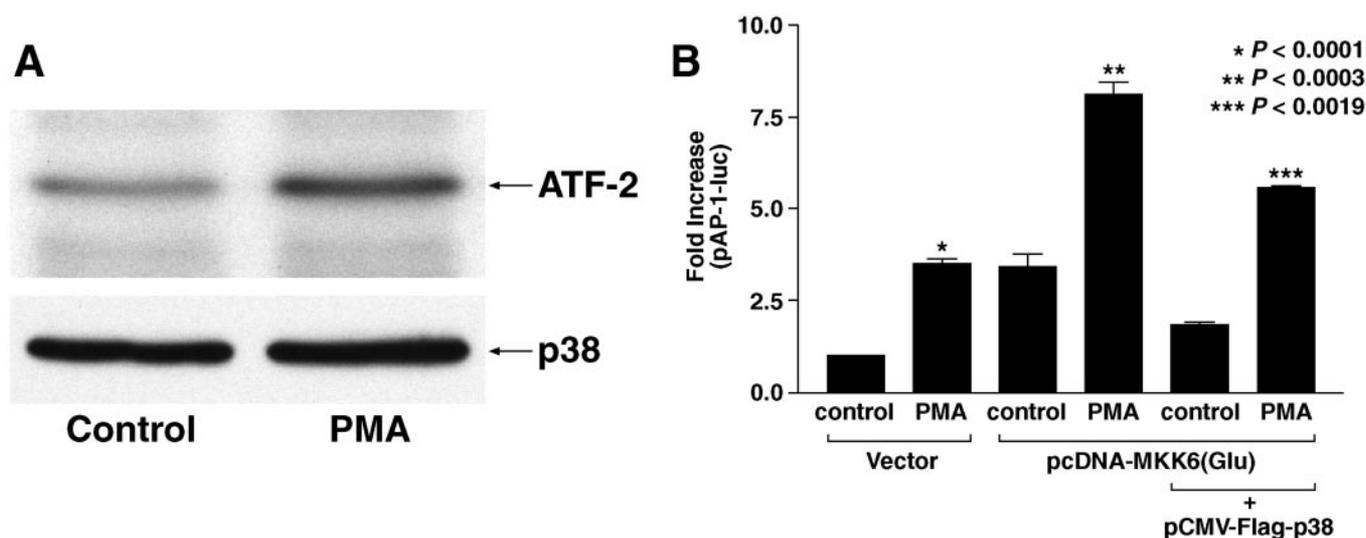


FIG. 6. Phorbol esters activate the p38 MAP kinase in RAW 264.7 macrophages and increase AP-1-dependent gene expression. *A*, cells were exposed to PMA for 15 min, and whole cell lysates were subjected to immunoprecipitation with p38 MAP kinase rabbit polyclonal antibody. *In vitro* kinase assays were performed using ATF-2 as the substrate. Western blot analysis for p38 MAP kinase was performed to confirm equal loading of the proteins. *B*, RAW 264.7 cells were transiently co-transfected with pAP-1-luc and either an empty vector or the constitutive active MKK6(Glu) vector with or without the dominant negative p38 expression vector. The total amount of plasmid DNA was equivalent in each sample by adding respective amounts of the empty vector. After 24 h, the cells were exposed to PMA for 6 h. Luciferase activity, which is normalized to total protein, is expressed as fold increase from control.

Xpress monoclonal antibody. Samples were separated by SDS-PAGE, and Western blot analysis was performed for c-Jun. We found that there was a significant increase in c-Jun interaction with His-TBP in cells expressing the constitutive active MKK6(Glu) (Fig. 5B). PMA alone did not increase this interaction in cells expressing the empty vector. We performed a similar experiment in cells cultured in the presence or absence of SB 203580 after the transient co-transfection. SB 203580 abrogated the effect of MKK6(Glu) on the interaction of c-Jun and His-TBP (Fig. 5C).

To complement the results of the data in Fig. 5, *B* and *C*, we co-transfected THP-1 cells with pcDNA-His-TBP and the constitutive active MKK6(Glu) either alone or with the dominant negative p38 expression vector. The results from these experiments corroborated the results found with SB 203580. The cells expressing both MKK6(Glu) and the dominant negative p38 expression vector had significantly decreased interaction of c-Jun and His-TBP compared with the cells expressing the constitutive active MKK6(Glu) alone (Fig. 5D). Taken together, these studies show that TBP activation in macrophages requires an active p38 MAP kinase. Furthermore, these studies show that TBP phosphorylation is necessary for the direct interaction of TBP with c-Jun and thus for AP-1-driven transcription.

To evaluate the necessity of p38 MAP kinase activity in TBP activation and subsequent AP-1-dependent gene expression, we performed similar studies in a different macrophage cell line. In contrast to THP-1 cells, PMA modestly activates the p38 MAP kinase in RAW 264.7 cells. Cells were exposed to PMA for 15 min, and whole cell lysates were subjected to immunoprecipitation with the p38 MAP kinase rabbit polyclonal antibody. *In vitro* kinase assays were performed using ATF-2 as the substrate. PMA significantly increased the phosphorylation of ATF-2 compared with control cells (Fig. 6A). Western blot analysis for the p38 MAP kinase confirmed equal loading of the immunoprecipitated proteins.

AP-1-driven transcription was determined by co-transfecting RAW 264.7 cells with pAP-1-luc and either an empty vector or the constitutively active MKK6(Glu) expression vector with or without the dominant negative pCMV-Flag-p38 plasmid. Cells were exposed to PMA for 6 h the day after the transient co-transfection. In cells expressing the empty vector, PMA signif-

icantly increased (~3.5 times control) AP-1-dependent luciferase activity. This activity was significantly augmented in cells expressing MKK6(Glu), which is more effective in activating the p38 MAP kinase, but in cells expressing both MKK6(Glu) and the dominant negative p38 expression vector, this luciferase activity was significantly inhibited (Fig. 6B). In PMA-stimulated RAW 264.7 cells, all MAP kinases (ERK, JNK (data not shown), and p38) are activated. Thus, PMA-stimulated RAW 264.7 cells will have increased c-Fos and c-Jun synthesis due to ERK- and JNK-mediated signaling, increased c-Jun phosphorylation due to JNK-mediated and possibly p38-mediated signaling, and increased TBP phosphorylation due to p38 MAP kinase activity. Take together, these studies show that the p38 MAP kinase phosphorylates TBP and that this phosphorylation is necessary for AP-1-driven transcription in macrophages.

DISCUSSION

We have previously shown that the p38 MAP kinase regulates NF- κ B-dependent gene expression by modulating the phosphorylation and subsequent activation of TBP (19, 20). In this study, we asked whether the p38 MAP kinase regulated the transcriptional activity of AP-1. We found that PMA alone was unable to drive the AP-1-dependent reporter gene in THP-1 cells. PMA activated both the ERK and JNK MAP kinases, but it did not activate the p38 MAP kinase. We found that cells expressing a constitutive active MKK6(Glu) had significantly increased AP-1-dependent gene expression in PMA-stimulated THP-1 cells, and SB 203580 abrogated the effect of MKK6(Glu). Expression of a constitutive active MKK6(Glu) resulted in the phosphorylation of a His-TBP fusion protein, and it increased direct interaction of His-TBP with c-Jun. PMA alone had no significant effect on either the phosphorylation of TBP or the interaction of TBP with c-Jun. In addition, cells expressing MKK6(Glu) had increased amounts of phosphorylated c-Jun. To further support these data, we used a different macrophage cell line (RAW 264.7) in which the p38 MAP kinase is activated by PMA. We found that AP-1-dependent gene expression was increased by PMA alone, but this activity was significantly increased in cells expressing MKK6(Glu), which is a more potent activator of the p38 MAP kinase. These findings suggest that in macrophages, the p38

MAP kinase regulates AP-1-driven transcription by modulating the activation of TBP.

The regulation of AP-1-dependent gene expression can occur at multiple levels after cell stimulation. AP-1 proteins are transcribed from immediate-early genes. The induction of *c-fos* transcription by MAP kinases occurs via a serum response element (28). After cell stimulation, the transcription factor Elk-1 is phosphorylated by both the ERK and JNK MAP kinases. This phosphorylation results in the formation of a ternary complex composed of Elk-1, the serum response element, and the serum response factor needed to initiate *c-fos* transcription (29, 30). Once translated, the c-Fos protein is subsequently able to translocate to the nucleus, form dimers with Jun proteins, and bind to DNA.

Unlike *c-fos*, the transcription of *c-jun* is dependent only on JNK MAP kinase activation. The *c-jun* promoter has a 12-O-tetradecanoylphorbol-13-acetate response element that is constitutively occupied by c-Jun/ATF-2 heterodimers that are each phosphorylated by JNK MAP kinase. This phosphorylation is required to induce transcription (31–35). AP-1 transcriptional activity, then, is dependent on both ERK and JNK MAP kinase-mediated signaling for c-Fos and c-Jun synthesis and c-Jun phosphorylation. Our data show that phorbol esters activate both the ERK and JNK MAP kinases in THP-1 cells but are unable to drive an AP-1-dependent gene.

The involvement of the p38 MAP kinase in AP-1-driven transcription has been suggested in several studies. In murine epidermal cells exposed to silica, inhibition of the p38 MAP kinase with SB 203580 resulted in reduction in AP-1 DNA binding (18). Another study has shown that certain p38 isoforms, in combination with ERK5, are necessary to activate the *c-jun* promoter in murine fibroblasts by a JNK-independent mechanism (36). The role of these isoforms, p38 α and p38 γ , however, was not evaluated in terms of AP-1 transcriptional activity. Our data suggest that an important role of the p38 MAP kinase in regulating AP-1-driven transcription occurs downstream of Jun and Fos protein synthesis and AP-1 DNA binding. We do, however, also show that the p38 MAP kinase phosphorylates c-Jun, which is necessary for AP-1-driven transcription.

AP-1 proteins have been shown to interact with both enhancer transcription factors, such as ATF-2 and NF- κ B (32, 37), and basal transcription factors, such as TFIIB and TBP (9–11). All Jun proteins interact with both TFIIB and TBP *in vitro*, and TBP is capable of interacting with c-Jun at both the N-terminal transcriptional activation and the C-terminal basic leucine zipper domains (11). The phosphorylation of c-Jun on its N-terminal domain is required for transcriptional activity, but this phosphorylation is not necessary for TBP/c-Jun interaction. It is unclear from this study, however, whether this interaction is required for AP-1 transcriptional activity *in vivo*.

The role of the p38 MAP kinase in regulating both NF- κ B and ATF-2 have been shown previously (19–21, 38, 39). In fact, we have shown that the p38 MAP kinase regulates NF- κ B transcriptional activity by modulating the phosphorylation of TBP. TBP is a required component in class I, II, and III promoters for the initiation of transcription (40–43), and the phosphorylation of TBP has been shown to be essential for the transcription of class II promoters (19, 20, 44). It is unlikely that either ATF-2 or NF- κ B is required in our system, because our luciferase reporter is driven only by AP-1. The novel aspect of the current study is that the transcriptional activity of AP-1, like NF- κ B, is regulated by the p38 MAP kinase through its modulation of TBP activation. Furthermore, the phosphorylation of TBP by the p38 MAP kinase is necessary for its interaction with c-Jun. Thus, an active p38 MAP kinase is necessary

in order for macrophages to express inflammatory genes dependent on AP-1 and/or NF- κ B.

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